

## Revisiting the morphology and phylogeny of *Lactifluus* with three new lineages from southern China

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**Abstract:** As a recent group mainly defined by molecular data the genus *Lactifluus* is in need of further study to provide insight into the morphological and molecular variation within the genus, species limits and relationships. Phylogenetic analyses of nuc rDNA ITS1-5.8S-ITS2 (ITS), D1 and D2 domains of nuc 28S rDNA (28S), and part of the second largest subunit of the RNA polymerase II (*rpb2*) (6–7 region) sequences of 28 samples from southern China revealed three new lineages of *Lactifluus*. Two of them are nested in a major clade that includes the type of *Lactifluus* and here is treated as two new sections: *L. sect. Ambicystidiati* and *L. sect. Tenuicystidiati*. *Lactifluus ambicystidiatus*, described here as a new species (= sect. *Ambicystidiati*), has both lamprocystidia and macrocystidia in the hymenium, a unique combination of features within Russulaceae. Furthermore, only remnants of lactiferous hyphae are present in *L. ambicystidiatus* and our results suggest that the ability to form a lactiferous system has been lost in this lineage. *Lactifluus sect. Tenuicystidiati* forms a strongly supported monophyletic group as a sister lineage to *L. sect. Lactifluus*. We recognize it based on the thin-walled macrocystidia and smaller ellipsoid spores with an incomplete reticulum compared with *L. sect. Lactifluus*. The former placement of *L. tenuicystidiatus* in the African *L. sect. Pseudogymnocarpi* is not supported. Using genealogical

concordance we recognize five phylogenetic species within *L. sect. Tenuicystidiati* and describe two of these as new, *L. subpruinus* and *L. tropicosinicus*. The third lineage, represented by *L. leoninus*, forms a sister group to *L. subg. Lactariopsis* sensu stricto. The three lineages provide further evidence for morphological features in *Lactifluus* being homoplasious. Some sections and species complexes are likely to be composed of more species and merit further investigations. Subtropical-tropical Asia is likely a key region for additional sampling.

**Key words:** *Lactarius*, lactiferous hyphae, Russulaceae, subtropical-tropical Asia, taxonomy

### INTRODUCTION

Traditional classification of Russulaceae assigned all agarics lacking milk to *Russula* Pers. and the milky ones to *Lactarius* Pers., although several names were proposed to divide milkcaps into different genera (Redeuilh et al. 2001). Recent molecular data have shown that *Lactarius* sensu lato is not monophyletic and species of the genus are spread in three separate lineages (Buyck et al. 2008). A new genus *Multifurca* Buyck & Hofstetter accordingly was described to accommodate a few atypical species previously treated in *Lactarius* and *Russula* (Buyck et al. 2008). The conservation of the name *Lactarius* with a conserved type, *L. torminosus* (Schaeff.: Fr.) Pers., made it possible to leave most of the previously described species in *Lactarius* (Buyck et al. 2010, Barrie 2011, Norvell 2011, McNeill et al. 2012) and to re-apply the name *Lactifluus* (Pers.) Roussel for the remainder of the milkcaps, typified by *Lactarius volemus* (Fr.: Fr.) Fr. (Buyck et al. 2010).

*Lactifluus* morphology is highly diverse. It includes species with veiled and unveiled, agaricoid and pleurotoid, lactarioid and russuloid sporophore forms. Macromorphologically it is not always clearly delimited from *Lactarius* and *Russula*. For example, *L. subg. Gerardii* (A.H. Sm.) Stubbe and *L. subg. Russulopsis* (Verbeken) Verbeken are strongly similar to *Lactarius* and *Russula*, respectively (Verbeken 2001, Stubbe et al. 2010, Verbeken et al. 2011). Microscopically the genus has more types of pileipellis and stipitipellis than *Lactarius* and *Multifurca*, varying from cutis to palisade, over trichoderm or trichopalisade, with or without ixo layers, with or without thick-walled elements, with or without dermatocystidia and including some deviating

types. The morphological richness is still growing with new representatives discovered from the tropics (van de Putte et al. 2009, Miller et al. 2012, Wang et al. 2012, Morozova et al. 2013).

Buyck et al. (2008) presented a three-locus phylogeny of *Lactifluus* (as “*Lactarius* 1”). It contained 15 species, belonging to the four subgenera proposed by Verbeken et al. (2011, 2012). Before and after Buyck et al. (2008) additional taxa from Africa, America and Asia were documented with molecular data, either using a single locus or a multigene approach (Henkel et al. 2000; Buyck et al. 2007; Stubbe et al. 2010; van de Putte et al. 2010, 2012; Wang et al. 2012; Morozova et al. 2013; de Crop et al. 2014a; Maba et al. 2014, 2015). These taxa represented all six currently described subgenera (Verbeken et al. 2011, 2012; Stubbe et al. 2012). The *Lactifluus* phylogeny, however, is far from complete. The circumscription of several sections, for example, *L.* sect. *Allardii* (Hesler & A.H. Sm.) de Crop, *L.* sect. *Aurantiiifolii* (Verbeken) Verbeken, *L.* sect. *Phlebonemi* (R. Heim ex Verbeken) Verbeken and *L.* sect. *Polysphaerophori* (Singer) Verbeken has not been tested using molecular data. Several morphology-based subgeneric taxa have been suggested to be paraphyletic using molecular phylogenetics (Buyck et al. 2008, Stubbe et al. 2010, van de Putte et al. 2010), but further sampling of species and genes are needed to confirm this. Molecular data are needed to clarify the relationships of species with still uncertain or isolated systematic positions (Wang and Verbeken 2006, Miller et al. 2012, Verbeken et al. 2012), for example, *L. subiculatus* S.L. Miller et al., *L. cocosmus* (van de Putte & de Kesel) van de Putte, *L. tenuicystidiatus* (X.H. Wang & Verbeken) X.H. Wang and several species described from South America (Singer 1975, 1984; Singer et al. 1983). An up-to-date classification is being prepared by de Crop et al. (2014b pers comm).

Compared with *Lactarius*, *Lactifluus* is more abundant and widely distributed in the tropics. In terms of endemism of infrageneric taxa and number of species described, tropical Africa has the highest diversity of *Lactifluus* (Verbeken and Walley 2011; Verbeken et al. 2011, 2012). Recent sampling in subtropical-tropical Asia (mainly focused on *L.* sect. *Lactifluus*, *L.* subg. *Gerardii* and *L.* subg. *Piperati* Verbeken) has demonstrated high diversity in this still largely unexplored continent (Stubbe et al. 2010, 2012; van de Putte et al. 2010, 2012; Wang et al. 2012; Morozova et al. 2013; de Crop et al. 2014a). The current study revisiting Asia with sampling in southern China added three new distinct lineages to *Lactifluus*. One of them concerns a species originally described from this region, *L. tenuicystidiatus*, whose exact placement within *Lactifluus* was left as an open

question when it was described (Wang and Verbeken 2006). In addition, this study provided more data to document the morphological and genetic diversity within *Lactifluus*.

## MATERIALS AND METHODS

**Sampling.**—Six samples of *L. ambicystidiatus* X.H. Wang, 23 of the *L. tenuicystidiatus* species complex and one of *L. aff. leoninus* (Verbeken & E. Horak) Verbeken, were used for morphological and (or) molecular study. These samples were collected from six provinces in southwestern and southern China. Three loci, nuc rDNA ITS1-5.8S-ITS2 (ITS), D1 and D2 domains of nuc 28S rDNA (28S), and part of the second largest subunit of the RNA polymerase II (*rpb2*) (6–7 region) were amplified and sequenced for 28 of them. ITS was amplified and sequenced for the holotype of *L. tenuicystidiatus*. To provide more data to the *Lactifluus* phylogeny, ITS, 28S and *rpb2* of two Asian samples of *L. aff. luteolus* also were sequenced (representing molecularly unsampled *L.* sect. *Phlebonemi*). Published sequences of 31 taxa from Buyck et al. (2007, 2008), Stubbe et al. (2010), Tedersoo and Pölme (2012), van de Putte et al. (2010, 2012), Wang et al. (2012) and Morozova et al. (2013) were in addition retrieved from GenBank (TABLE I). These sequences were chosen to cover the representatives of *Lactifluus* with at least two of the three loci used in this study. They involved five of the six subgenera and eight of the 15 sections recognized in *Lactifluus*. Among the 31 taxa with sequences retrieved from GenBank, six lack sequences from one of the three loci: *Lactifluus* aff. *leoninus* and *L. rugatus* (Kühner & Romagn.) Verbeken lack ITS sequences, *L. emergens* lacks 28S sequence and *L. chrysocarpus*, *L. igniculus* and *L. leoninus* lack *rpb2* sequences.

**Morphological study.**—Macro- and microscopical descriptions are based on fresh and dried materials, respectively. Spores were observed in Melzer’s reagent and measured in side view, excluding ornamentation and apiculus. Statistic of spore measurements follows Yang (2000). All other microscopical structures were observed on slides made with 5% KOH and mounted with Congo red (aqueous reagent). All drawings, except those of the spores, were made with a drawing tube installed on a Nikon E400 microscope. Drawings of spores were made by hand. Terminology in descriptions of pileipellis follows Verbeken (1998a). Color codes are from Kornerup and Wanscher (1961).

**DNA extraction, PCR amplifications and sequencing.**—Total genomic DNA was extracted from dried pieces of pileus with lamellae with a CTAB protocol (Doyle and Doyle 1987). The primers ITS1-F or ITS1, and ITS4, LR0R and LR5, and bRPB2-6f and fRPB2-7cR were used to amplify the ITS region, part of the 28S, and the region between conserved domains 6 and 7 of *rpb2*, respectively (White et al. 1990; Liu et al. 1999; R. Vilgalys lab, <http://www.biology.duke.edu/fungi/mycolab/primers.htm>). PCR amplification was performed with Takara® or Takara Ex® DNA polymerase (Dalian, China) using the following protocol (25 µL reaction mixture): 2.5 µL buffer, 2.5 µL 0.1% BSA, 0.5 µL 10 mM

TABLE I. Taxa and collections used for molecular phylogenetic analyses in this study<sup>a</sup>

Species	Strain (herbarium)	Geographical origin	GenBank accession No.		
			ITS	28S	<i>rpb2</i>
<i>Lactarius camphoratus</i>	U. Eberhardt 04.09.2004-5 (UPS)	Sweden	DQ422009	DQ422009	DQ421933
<i>Lactarius chiapanensis</i>	V.M. Bandala 4374A (GENT)	Mexico	GU258297	GU265580	GU258316
<i>Lactarius lignyotus</i>	U. Eberhardt 06.09.2003-5 (UPS)	Sweden	DQ421993	DQ421993	DQ421926
<i>Lactarius pubescens</i>	U. Eberhardt 15.09.2002-2 (UPS)	Sweden	DQ421996	DQ421996	DQ421929
<i>Lactifluus ambicystidiatus</i>	H.J. Li 140803-50 (KUN)	Yunnan, China	KR908670	KR908672	KR908674
<i>Lactifluus ambicystidiatus</i>	H.J. Li 140805-26 (KUN)	Yunnan, China	KR908671	KR908673	KR908675
<i>Lactifluus ambicystidiatus</i>	J.P. Zhang 72 (KUN)	Yunnan, China	KC154095	KC154121	KC154147
<i>Lactifluus ambicystidiatus</i>	L.P. Tang 1051 (KUN, holotype)	Yunnan, China	KC154096	KC154122	KC154148
<i>Lactifluus chrysocarpus</i>	E. Popov LE253907 (LE)	Vietnam	JX442761	JX442761	—
<i>Lactifluus clarkeae</i>	M. Noordeloos 2004002	Australia	HQ318282	HQ318205	HQ328933
<i>Lactifluus clarkeae</i>	M. Noordeloos 2004122	Australia	HQ318284	HQ318207	HQ328935
<i>Lactifluus corrugis</i>	A. Verbeken 04-209 (GENT)	USA	JN388977	JN388998	JN375601
<i>Lactifluus crocatus</i>	K. Van de Putte 08-034 (GENT)	Thailand	HQ318243	HQ318151	HQ328888
<i>Lactifluus deceptivus</i>	A. Verbeken 04-181 (GENT)	USA	DQ422020	DQ422020	DQ421935
<i>Lactifluus densifolius</i>	B. Buyck 12.1994 (PC)	Burundi	—	DQ421980	DQ421920
<i>Lactifluus dissitus</i>	A. Verbeken 09-134 (GENT)	Sikkim	JN388978	JN389026	JN375628
<i>Lactifluus distantifolius</i>	D. Stubbe 07-461 (GENT)	Thailand	HQ318223	HQ318124	HQ328866
<i>Lactifluus edulis</i>	A. Verbeken 99-041 (GENT)	Zimbabwe	AY606973	DQ421977	DQ421916
<i>Lactifluus emergens</i>	A. Verbeken 99-005 (GENT)	Zimbabwe	AY606979	—	DQ421919
<i>Lactifluus gerardii</i>	A. Verbeken 05-355 (GENT)	USA	GU258253	GU265615	GU258352
<i>Lactifluus glaucescens</i>	A. Verbeken 04-202 (GENT)	USA	HQ318280	HQ318203	HQ328932
<i>Lactifluus genevievae</i>	G. Gates/D. Ratkowsky 17-02-05 (GENT)	Australia	GU258294	GU265657	GU258397
<i>Lactifluus hygrophoroides</i>	A. Verbeken 05-251 (GENT)	USA	HQ318285	HQ318208	HQ328936
<i>Lactifluus igniculus</i>	O. Morozova LE262983 (LE)	Vietnam	JX442759	JX442759	—
<i>Lactifluus inversus</i>	AB63 (GENT)	Guinea	AY606976	DQ421978	DQ421917
<i>Lactifluus leoninus</i>	L-GN2a (environmental sample)	Papua New Guinea	JX316730	JX316730	—
<i>Lactifluus</i> aff. <i>leoninus</i>	D. Stubbe 07-454 (GENT)	Thailand	—	JN388989	JN375592
<i>Lactifluus</i> aff. <i>leoninus</i>	X.H. Wang 3405 (KUN)	Yunnan, China	KC154097	KC154123	KC154149
<i>Lactifluus longisporus</i>	A. Verbeken 99-197 (GENT)	Zimbabwe	DQ421971	DQ421971	DQ421910
<i>Lactifluus</i> aff. <i>luteolus</i>	X.H. Wang 2997 (KUN)	Shandong, China	KC154098	KC154124	KC154150
<i>Lactifluus</i> aff. <i>luteolus</i>	X.H. Wang 2980 (KUN)	Incheon, Korea	KC154099	KC154125	KC154151
<i>Lactifluus madagascariensis</i>	B. Buyck 99-409 (PC)	Madagascar	AY606977	DQ421975	DQ421914
<i>Lactifluus parvigerardii</i>	X.H. Wang 2415 (KUN)	Guizhou, China	JF975641	JF975642	JF975643
<i>Lactifluus pelliculatus</i>	B. Buyck 00-1335 (PC)	Madagascar	AY606978	DQ421974	DQ421913
<i>Lactifluus petersenii</i>	A. Verbeken 05-300 (GENT)	USA	GU258281	GU265642	GU258382
<i>Lactifluus piperatus</i>	U. Eberhardt 09.08.2004-6 (UPS)	Sweden	DQ422035	DQ422035	DQ421937
<i>Lactifluus pseudoluteopus</i>	A. Verbeken 04-129 (GENT)	Thailand	HQ318286	HQ318210	HQ328938
<i>Lactifluus rubroviolascens</i>	B. Buyck 97.266 (PC)	Madagascar	AY606985	DQ421972	DQ421911
<i>Lactifluus rugatus</i>	PA2010R (GENT)	Greece	—	JN388992	JN375565
<i>Lactifluus subpruinosis</i>	X.H. Wang 3036 (KUN)	Anhui, China	KC154108	KC154134	KC154160
<i>Lactifluus subpruinosis</i>	Y.C. Li 1011 (KUN)	Fujian, China	KC154112	KC154138	KC154164
<i>Lactifluus subpruinosis</i>	F. Li 330 (KUN)	Guangdong, China	KC154106	KC154132	KC154158
<i>Lactifluus subpruinosis</i>	X.H. Wang 3131 (KUN, holotype)	Guangdong, China	KC154109	KC154135	KC154161
<i>Lactifluus subpruinosis</i>	Q. Zhao 282 (KUN)	Yunnan, China	KC154107	KC154133	KC154159
<i>Lactifluus subpruinosis</i>	X.H. Wang 3489 (KUN)	Yunnan, China	KC154110	KC154136	KC154162
<i>Lactifluus subpruinosis</i>	X.H. Wang 3514 (KUN)	Yunnan, China	KC154111	KC154137	KC154163
<i>Lactifluus tenuicystidiatus</i>	B. Feng 824 (KUN)	Yunnan, China	KC154101	KC154127	KC154153
<i>Lactifluus tenuicystidiatus</i>	J.P. Zhang 119 (KUN)	Yunnan, China	KC154102	KC154128	KC154154



TABLE I. Continued

<i>Lactifluus tenuicystidiatus</i>	X.H. Wang 1137 (KUN, holotype)	Yunnan, China	KP347667	—	—
<i>Lactifluus</i> aff. <i>tenuicystidiatus</i>	X.H. Wang 3490 (KUN)	Yunnan, China	KC154103	KC154129	KC154155
<i>Lactifluus</i> aff. <i>tenuicystidiatus</i>	X.H. Wang 3513 (KUN)	Yunnan, China	KC154104	KC154130	KC154156
<i>Lactifluus</i> aff. <i>tenuicystidiatus</i>	X.H. Wang 3515 (KUN)	Yunnan, China	KC154105	KC154131	KC154157
<i>Lactifluus tropicosinicus</i>	L.P. Tang 1011 (KUN)	Yunnan, China	KC154113	KC154139	KC154165
<i>Lactifluus tropicosinicus</i>	Q. Cai 52 (KUN)	Yunnan, China	KC154114	KC154140	KC154166
<i>Lactifluus tropicosinicus</i>	X.T. Zhu 477 (KUN)	Yunnan, China	KC154119	KC154145	KC154171
<i>Lactifluus tropicosinicus</i>	Y.C. Li 1878 (KUN)	Yunnan, China	KC154120	KC154146	KC154172
<i>Lactifluus tropicosinicus</i>	Y.C. Li 1879 (KUN, holotype)	Yunnan, China	KP347668	KP347669	KP347670
<i>Lactifluus</i> aff. <i>tropicosinicus</i>	X.H. Wang 3449 (KUN)	Yunnan, China	KC154115	KC154141	KC154167
<i>Lactifluus</i> aff. <i>tropicosinicus</i>	X.H. Wang 3450 (KUN)	Yunnan, China	KC154116	KC154142	KC154168
<i>Lactifluus</i> aff. <i>tropicosinicus</i>	X.H. Wang 3451 (KUN)	Yunnan, China	KC154117	KC154143	KC154169
<i>Lactifluus</i> aff. <i>tropicosinicus</i>	X.H. Wang 3512 (KUN)	Yunnan, China	KC154118	KC154144	KC154170
<i>Lactifluus vellereus</i>	U. Eberhardt 20.09.2004-22 (UPS)	Sweden	DQ422034	DQ422034	DQ421936
<i>Lactifluus velutissimus</i>	A. Verbeken 99-085 (GENT)	Zimbabwe	AY606982	DQ421973	DQ421912
<i>Lactifluus volemus</i>	90804-5 (GENT)	Sweden	JN388959	JN389010	JN375612
<i>Multifurca furcata</i>	R. Halling 7804 (NY)	Costa Rica	DQ421994	DQ421994	DQ421927
<i>Multifurca ochricompacta</i>	B. Buyck 02.107 (PC)	USA	DQ421984	DQ421984	DQ421940
<i>Multifurca zonaria</i>	D.E. Desjardin 7442 (SFSU, PC)	Thailand	DQ421990	DQ421990	DQ421942
<i>Russula camarophylla</i>	P.A. Moreau 01081108 (PC)	France	DQ421982	DQ421982	DQ421938
<i>Russula cyanoxantha</i>	U. Eberhardt 29.09.2002-2 (UPS)	France	DQ422033	DQ422033	DQ421970
<i>Russula</i> cf. <i>delica</i>	U. Eberhardt 24.08.2004-20 (UPS)	Sweden	DQ422005	DQ422005	DQ421950
<i>Russula emetica</i>	U. Eberhardt 05.10.2003-11 (UPS)	Sweden	DQ421997	DQ421997	DQ421943
<i>Russula pallescens</i>	P. Larsen 146/2002 (TUR)	Norway	DQ421987	DQ421987	DQ421941

<sup>a</sup>Sequences produced in the present study in boldface. ITS = nuc rDNA ITS1-5.8S-ITS2; 28S = D1 and D2 domains of nuc 28S rDNA; *rpb2* = part of the second largest subunit of the RNA polymerase II (6–7 region).

dNTPs, 0.5 µL 10 µM of forward and reverse primers, 0.25 µL 5U/µL Taq polymerase, 0.1–1 µL total DNA solution and 18 µL ddH<sub>2</sub>O. For amplification of *rpb2*, 0.25–0.5 µL 25 mM MgCl<sub>2</sub> was added when PCR products were not sufficient. The following PCR programs were used: 5 min at 94.0 °C, 35 cycles of 1 min at 94.0 °C, 1.5 min at 48.0 °C, or 50 °C, or 53.0 °C, and 2 min at 72.0 °C, and a final extension of 72.0 °C for 10 min. In a few cases, when amplification products were either faint or direct sequencing failed, PCR products were cloned with the Takara® pMD™18T cloning kit (Dalian, China) following the manufacturer's instructions. Colonies were screened for the presence of the desired products with primers M13F and M13R or the original PCR primers. At least two clones with desired PCR products were sequenced. When sequences from the different clones differed only in base substitutions, they were merged into one sequence by replacing the substitutions with degenerate bases. For clones with different INDELs (insertion and deletion), a sequence of only one clone was used (with bases degenerated if any). Sequences of different clones with different INDELs proved to produce highly similar results in the phylogenetic analyses.

**Molecular phylogenetic analyses.**—Alignments were made with MUSCLE 3.6 (Edgar 2004) and manually adjusted in

BioEdit. To test the effect of ambiguously aligned sections of the ITS alignment on the phylogenetic results, two datasets were prepared: (i) the inclusive dataset, with all characters kept, and (ii) the exclusive dataset, with characters selected by Gblocks 0.91b (Castresana 2000) with the following settings: minimum number of sequences of a conserved position (35), minimum number of sequences of a flank position (58), maximum number of contiguous non-conserved positions (eight), minimum length of a block (six) and allowed gap positions (one-half). Maximum likelihood (ML) analyses were conducted to compare the difference of the two datasets. To ensure that homology in the ITS alignment was being properly defined, topology and ML bootstrap proportions (ML-BP) produced by the ITS datasets were compared with those of the 28S-*rpb2* dataset, which has much fewer ambiguous aligned sections in the matrix, tree provided (SUPPLEMENTARY FIG. 1). The only *rpb2* intron (76 bp long), which was hard to align, was excluded entirely in the phylogenetic analyses.

Before combined analyses congruence among the ITS, 28S and *rpb2* datasets were determined by visually comparing the ML-BP resulting from analyses of the three individual alignments for the same set of taxa. A conflict was assumed

to be significant when two different relationships (one monophyletic and the other non-monophyletic) for the same set of taxa were both supported with ML-BP  $\geq 70\%$ . In the ML analyses of the three individual gene regions, the data were not partitioned.

To first determine the generic position of the target taxa within Russulales, ML analysis of a 28S-*rpb2* combined dataset was conducted with the nine representatives of the russuloid clade (= Russulales of Miller et al. 2006) (Lutzoni et al. 2004) as outgroups (SUPPLEMENTARY FIG. 1). After the phylogenetic position of the target taxa in *Lactifluus* was confirmed, ML and Bayesian Inference (BI) analyses were performed to construct the phylogeny of *Lactifluus*, using three representatives of *Lactarius* sensu novo, three of *Multifurca* and five of *Russula* as outgroups. ML analyses were conducted in RAxML 7.2.6 (Stamatakis 2006) and BI in MrBayes 3.2.1 (Ronquist et al. 2012). Two partitioning strategies were used to analyze the combined dataset in both ML and BI analyses: (i) ITS, 28S, *rpb2*; and (ii) ITS1-ITS2, 5.8S, 28S, *rpb2* first and second codon positions, and *rpb2* third codon position. ML analyses applied the rapid bootstrapping algorithm with 1000 replicates, followed by a ML tree search. For BI analyses, the best-fit model of nucleotide substitution was selected by the hierarchical likelihood ratio tests in MrModeltest 2.3 using PAUP\* 4.0 beta 10 (Swofford 2002, Nylander 2004). The BI analyses were conducted using four runs with four chains each for  $1 \times 10^7$  generations sampling every 100th tree. Runs were terminated when the average standard deviation of split frequencies went below 0.01 and ESS (effective sampling size) values were  $> 200$ . A majority rule consensus tree was built after discarding trees from a 25% burn-in. Trees generated by the two analyses were viewed and exported in FigTree 1.3.1.

To recognize species within *L. sect. Tenuicystidiati*, genealogical concordance phylogenetic species recognition (GCPSR; Taylor et al. 2000, Dettman et al. 2003) was followed. Independent evolutionary lineages were determined by comparing the groupings of individuals from each of the three locus genealogies. A clade was taken as an independent lineage if its monophyly was highly supported by both ML-BP ( $\geq 70\%$ ) and posterior probability of BI analysis (BI-PP) ( $\geq 0.95\%$ ) in at least one locus genealogy and was not contradicted in any other genealogy. When deciding which independent lineage represented phylogenetic species, exhaustive classification was followed. That is to say a lineage would be treated as a phylogenetic species if it did not leave any adjacent individual(s) unclassified. Otherwise the node would be traced down from that individual until all individuals were included in an evolutionary lineage (Dettman et al. 2003).

## RESULTS

**Phylogeny and species recognition.**—Twenty-nine new sequences of the ITS region, 28 of the partial 28S and *rpb2* genes were generated from 29 *Lactifluus* samples. For the holotype of *L. tenuicystidiatus*, only the ITS sequence was obtained. The inclusive ITS

dataset included 839 characters: 317 bp of ITS1 (complete), 157 bp of 5.8S and 365 bp of ITS2 (complete). The program Gblocks 0.91b retained 41% of the original positions of ITS (= 350 bp). The 28S and *rpb2* (with intron) alignments included 955 bp and 770 bp, respectively. The combined three-locus matrix is available at TreeBASE under accession no. S14663 (ITS: 1-839, 28S: 840-1794, *rpb2*: 1795-2564).

The ML analysis of the Russulales 28S-*rpb2* dataset confirmed the monophyly of *Lactifluus*, *Lactarius* sensu novo, *Multifurca* and *Russula* (SUPPLEMENTARY FIG. 1). The ML phylogenies produced from the inclusive and exclusive ITS datasets did not show any supported conflict, but the inclusive dataset gave higher support to many clades (SUPPLEMENTARY FIGS. 2, 3). For instance, for the five phylogenetic species recognized in *L. sect. Tenuicystidiati* (see below), the inclusive dataset recognized four of them whereas the exclusive dataset only recognized three. Moreover, the inclusive dataset gave higher support than the exclusive dataset for two of the species. Also the monophyly of *Lactifluus* (ML-BP = 91%) and the outgroup (ML-BP = 91%) received much higher support from the inclusive dataset than from the exclusive. The exclusive dataset, however, did resolve the *Ambicystidiatus* clade with *L. sect. Lactifluus*, *L. subg. Gerardii* and *L. sect. Tenuicystidiati*, in agreement with the analysis of the Russulales 28S-*rpb2* dataset, but this grouping did not have support. Compared with the exclusive dataset, the ambiguously aligned regions in the inclusive dataset did not seem to add noise to the analyses but improved the supports for several branches. Moreover, although the inclusive ITS dataset produced a different topology from that of the Russulales 28S-*rpb2* dataset, they did not differ significantly on the supported branches. The tree produced by the inclusive ITS-28S-*rpb2* dataset had almost the same topology as the Russulales 28S-*rpb2* dataset and the supporting values were comparable. The inclusive ITS dataset therefore was used for further analyses.

No supported conflict was revealed between the individual ITS, 28S and *rpb2* phylogenies and the three datasets therefore were combined for final phylogenetic analyses. The GTR+I+G model was selected as the best fit for the combined dataset. ML and BI analyses of the data partitioned under the two different strategies produced identical topologies with highly similar support values. Therefore only the ML tree produced using the ITS, 28S, *rpb2* partitioning strategy is presented, with BI-PP values shown on the branches (FIG. 1). ML and BI analyses of the ITS-28S-*rpb2* combined dataset produced highly resolved phylograms. The ESS values of the four runs of the BI analysis were 570.93–669.27. The

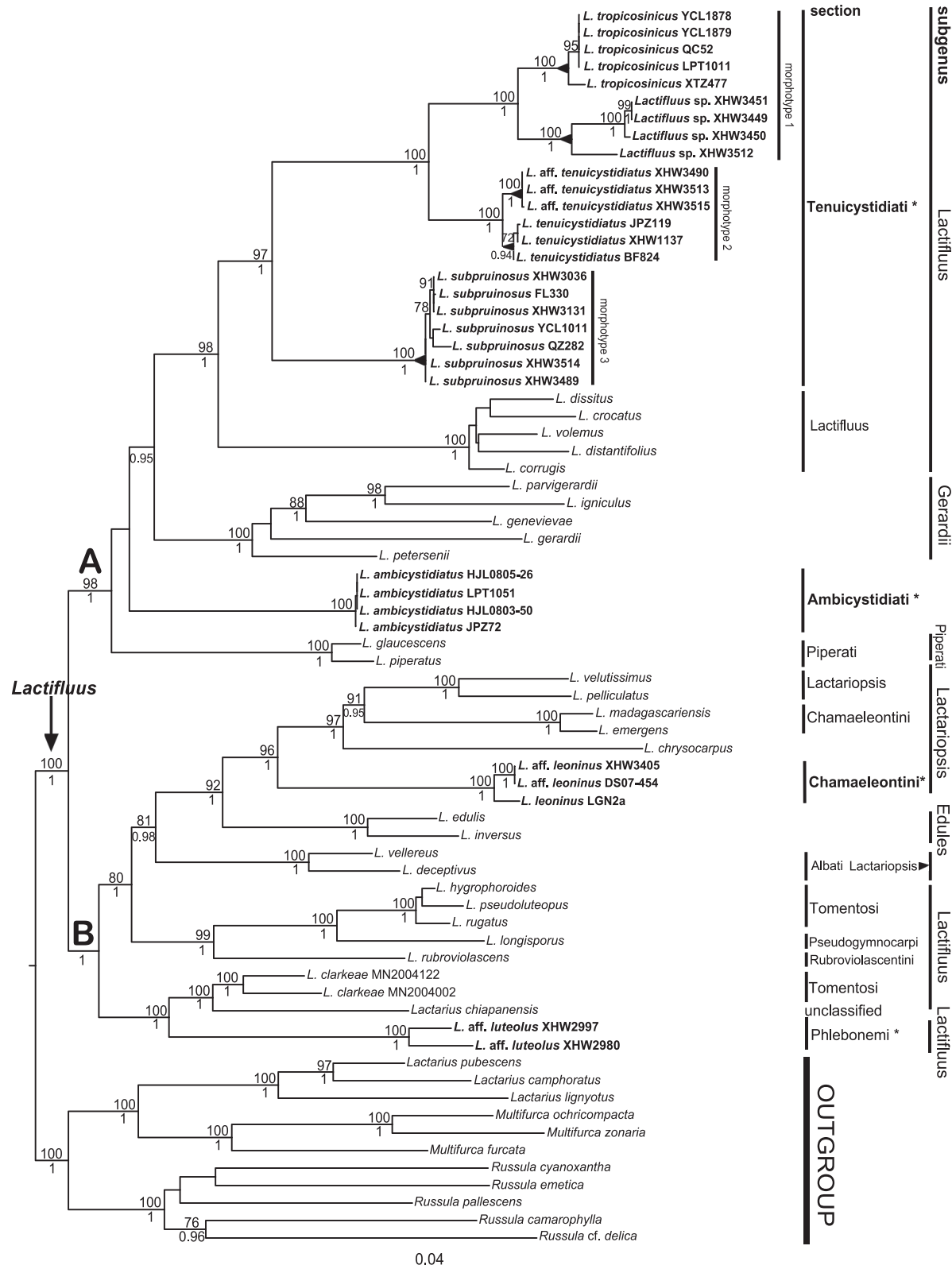


FIG. 1. Maximum likelihood phylogram of representatives of *Lactifluus* produced by combined analyses of ITS, 28S and *rpb2* sequences, rooted with 11 taxa of *Lactarius*, *Multifurca* and *Russula*. Maximum likelihood bootstrap proportions higher than 70% and Bayesian inference posterior probabilities higher than 95% are indicated above and below the internodes. Infrageneric classification follows Verbeken (2011, 2012) and Stubbe (2012). Taxa in boldface and clades marked with





FIG. 2. Basidiocarps of *Lactifluus*. a. *Lactifluus ambicystidiatus* (KUN F88179). b. *L. aff. leoninus* (KUN F75811). c. *L. subpruinosis* (KUN F73639, HOLOTYPE). d. *L. subpruinosis* (KUN F75812). e. *L. tenuicystidiatus* (KUN F74709). f. *L. tropicosinicus* (KUN F59627, HOLOTYPE).

topologies produced by the ML and BI analyses are nearly identical, only with minor differences on some terminal clades. BI-PP are similar or in some cases relatively higher than ML-BP values. The topologies regarding the involved taxa are comparable with those of Buyck et al. (2008) and Stubbe et al. (2010).

Two major clades (A, B) were inferred within *Lactifluus* (FIG. 1). Clade A included the type species of the genus, *L. volemus*. Samples of this major clade are exclusively from continents outside tropical Africa. Two of the target groups, *L. ambicystidiatus* and *L. sect. Tenuicystidiati*, were nested in this clade. Twenty-two samples with the general morphology of *L. sect. Tenuicystidiati* formed a highly supported subclade with ML-BP = 97% and BI-PP = 1.00. This subclade formed a sister group to *L. sect. Lactifluus* (the *L. volemus* species complex) (ML-BP = 98%, BI-PP = 1.00). Four samples of *L. ambicystidiatus* formed a distinct separate lineage. Its phylogenetic relationship with the other subclades in Clade A, however, was not well supported. The holotype of *L. tenuicystidiatus* was represented only by an ITS sequence, but its

phylogenetic placement was confirmed by the conspecific samples (JPZ119, BF824). *Lactifluus igniculus* lacks the *rpb2* sequence. This species formed a long branch in *L. subg. Gerardii* and its phylogenetic position should be tested using more genes.

Clade B only received significant support values in BI analysis (BI-PP = 1.00). ML analysis produced a support value of 55%. Within this major clade two well-supported subclades were obtained. One of them included the third target group, the *Leoninus* lineage. It formed a strongly supported sister group to a clade of tropical African *L. subg. Lactariopsis* sensu stricto and tropical Asian *L. chrysocarpus* E.S. Popov & O.V. Morozova. This subclade (subgenera *Lactariopsis* and *Edules*) is rich in tropical African samples. The other subclade included the two samples of *L. aff. luteolus* sequenced in this study. The sample of *L. aff. leoninus* (DS07-454) lacks ITS sequence and *L. leoninus* lacks *rpb2* but based on 28S these are very closely related or conspecific (SUPPLEMENTARY FIG. 4). *Lactifluus rugatus* lacks ITS, but based on 28S and *rpb2* sequences it formed a strongly supported clade with

←

asterisks are target or sequenced taxa of this study. Initials of the sample numbers correspond to the collectors (TABLE I). Triangles at nodes indicate the five phylogenetic species of *L. sect. Tenuicystidiati* recognized. Three morphotypes are recognized within *L. sect. Tenuicystidiati*, among which morphotype 1 and 2 cover two phylogenetic species, respectively.

*L. hygrophoroides* and *L. pseudoluteopus* (SUPPLEMENTARY FIG. 1). The singleton of *L. chrysocarpus* formed a long branch. Its phylogenetic position needs additional study.

By using a genealogical approach for phylogenetic species recognition (PSR; Taylor et al. 2000, Dettman et al. 2003), five phylogenetic species were recognized within *L. sect. Tenuicystidiati* (FIG. 1). All species were supported by ML-BP = 100% and BI-PP = 1.00, except for *L. tenuicystidiatus*, supported by ML-BP = 72% and BI-PP = 0.94. Macroscopic studies showed three morphological types, which correspond to three reciprocal monophyletic clades: morphotype 1 (FIG. 2 f), characterized by a pale yellow, subvelvety pileus and moderately spaced lamellae; morphotype 2 (FIG. 2e), similar to morphotype 1 but with distant lamellae and more robust basidiocarps; morphotype 3 (FIG. 2c, d), characterized by a reddish brown and velvety pileus and crowded lamellae. Morphotypes 1 and 2 include two phylogenetic species each. One of the two phylogenetic species of morphotype 2 represents authentic *L. tenuicystidiatus*, including the holotype of *L. tenuicystidiatus*, XHW1137. Morphotype 3 and one of the phylogenetic species under morphotype 1 are described as two new species, *L. subpruinus* X.H. Wang and *L. tropicosinicus* X.H. Wang (see below). Lacking clear morphological difference and sufficient sampling, the other two phylogenetic species were tentatively left as cryptic species of the named species.

#### TAXONOMY

Morphological and molecular data lead to the discovery of three new species, *L. ambicystidiatus*, *L. subpruinus* and *L. tropicosinicus*, and two new sections in *Lactifluus*, represented by *L. ambicystidiatus* and *L. tenuicystidiatus*, respectively. The new species and sections are proposed here. A description of the Chinese sample of *L. aff. leoninus* is also provided to highlight the morphological differences from the authentic *L. leoninus* described from Papua New Guinea.

***Lactifluus ambicystidiatus*** X.H. Wang, sp. nov.

FIGS. 2a, 3

Mycobank MB801945

**Typification:** CHINA. YUNNAN PROVINCE: Yongping County, roadside from Yongping to Baoshan, 25°26.598'N 99°25.423'E, 2087 m, on ground, 31-VII-2009, *L.P. Tang 1051* (KUN F57008, HOLOTYPE).

**Etymology:** named after the two types of hymenophoral true cystidia.

**Diagnosis:** *Lactifluus ambicystidiatus* is clearly distinguished by the pale cream-colored pileus, the hymenium with macrocystidia and lamprocystidia, and the tissue lacking a well-developed lactiferous network.

Basidiocarps large, stout, fleshy, brittle. Pileus 15–20 cm diam, depressed in center; surface velvety, whitish with pale orange or cream tinge, or pale yellow with faint pinkish tinge, locally paler. Context 5–7 mm thick in the pileus, whitish, not discoloring when bruised. Lamellae 4–5 mm wide, subdistant to distant, decurrent, sub-whitish to dull yellowish, sometimes forking. Stipe 5–6 × 2–3 cm, equal or tapering downward, stout, solid, velvety, concolorous with the lamellae or pileus. Latex milk white.

Spores (5.5–)6.0–7.5(–8.0) × (4.5–)5.0–6.0 μm ( $Q = [1.17–]1.18–1.35[–1.40]$ ,  $Q = 1.27 \pm 0.05$ ) (100/3/3), ellipsoid; ornamentation up to 0.3 μm high, composed of irregular warts and short ridges rarely connected; plage not amyloid or sometimes centrally amyloid. Basidia 50–60 × 7–9 μm, clavate, four-spored. Macrocystidia and lamprocystidia both present. Pleuromacrocystidia thin-walled, lanceolate to subcylindrical, often with mucronate apex, 60–100(–120) × 6–9(–10) μm, with dense needle-like contents, embedded in hymenium, arising either from the same level as basal septa of basidia or deeply from the subhymenium or trama, hardly projecting beyond the layer of basidia. Pleurolamprocystidia lanceolate, narrowly cylindrical, usually tapering upward and with very acute apex, 70–120(–140) × 7–10 μm, with thick walls (2–3 μm), embedded in hymenium, not projecting or projecting 10–30 μm beyond the layer of basidia, arising deeply from subhymenium or trama. Pleuropseudocystidia absent. Lamellar edge sterile; cheilomacrocystidia and cheilolamprocystidia common, smaller than the respective pleurocystidia. Hymenophoral trama with sphaerocytes, lacking typical rosettes. Pileipellis a lamprotrichopalisade; elements of suprapellis thick-walled (1 μm), up to 300 μm long, 4–5 μm thick, awl-shaped, with acute apex, often secondarily septate; subpellis composed of irregular or isodiametric cells not forming a regular layer, up to 80 μm thick; terminal of lactifers often embedded in hairs of suprapellis. Stipitipellis a lamprotrichoderm; hairs in suprapellis up to 100 μm long, base 5–6 μm thick, awl-shaped, thick-walled (1–1.5 μm); subpellis compact; thick-walled hyphae often originating deeply from the trama of stipe. Lactifers poorly developed, 5–7 μm thick, not branching, not forming pseudocystidia toward hymenium, often forming terminals in trama or near the surface of pileipellis and stipitipellis. Trama of pileus and stipe with typical rosettes.

**Specimens examined:** CHINA. SICHUAN PROVINCE: Pujiang County, Daxing, 30°14'N 103°25'E, 650 m, on





FIG. 3. *Lactifluus ambicystidiatus* (HOLOTYPE). a. Spores. b. Hymenium. c. Thin-walled pleuromacrocyttidia. d. Pleurolamprocystidia. e. Lamellar edge. f. Pileipellis. g. Stipitipellis. Bars: a = 5  $\mu$ m. b–g = 25  $\mu$ m.

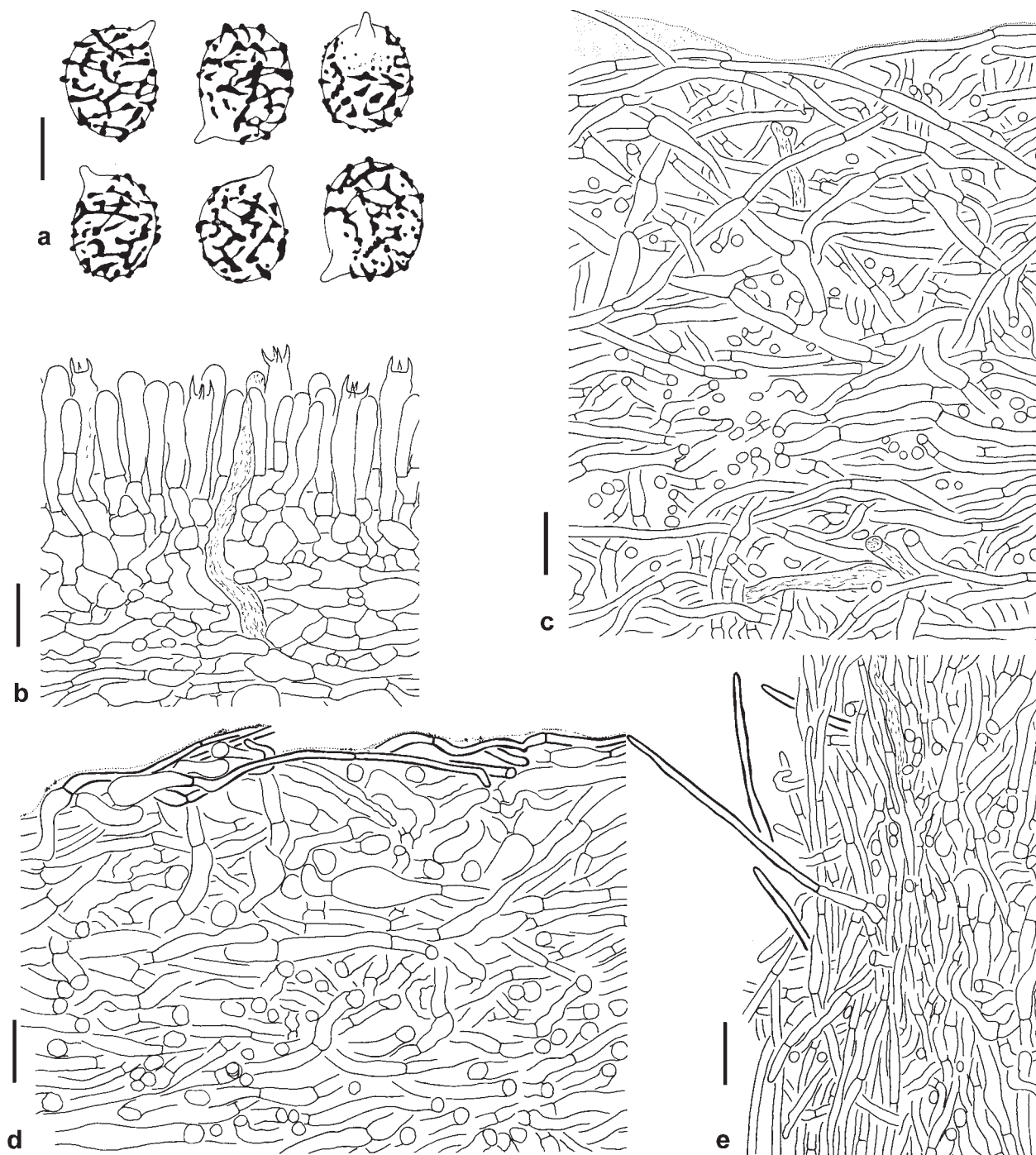


FIG. 4. *Lactifluus* aff. *leoninus* (KUN F75811). a. Spores. b. Hymenium. c. Pileipellis in young stage. d. Pileipellis in mature stage. e. Stipitipellis. Bars: a = 5  $\mu$ m. b–e = 25  $\mu$ m.

ground, 26-VII-1985, *M.S. Yuan* 1017 (KUN F15848); YUNNAN PROVINCE: Jianchuan County, Mt. Qianshi, 26°32'19"N 99°53'20"E, 2370 m, on ground in mixed forest with *Pinus yunnanensis*, 7-IX-2009, *J.P. Zhang* 72 (KUN F59005); Tengchong County, Guyong, 1800 m, 22-VII-1989, *W.K. Zheng* 79066 (KUN F4836); Tenchong, Qushi, Shuanghe village, on ground in mixed forest with *Pinus*

*yunnanensis* and fagaceous trees, 1760 m, 3-VIII-2014, *H.J. Li* 140803-50 (KUN F88179); Tenchong, Tengyue, Menlian village, on ground in mixed forest with *Pinus yunnanensis* and fagaceous trees, 1760 m, 5-VIII-2014, *H.J. Li* 140805-26 (KUN F88180).

*Notes:* *Lactifluus ambicystidiatus* is one of the most atypical species of milk caps. Although latex is absent

from some species of *Lactifluus* (Verbeken 2001, Buyck et al. 2007), *L. ambicystidiatus* is the first member in *Lactifluus* that has a highly degenerate lactiferous system with only remnants of lactifers. It does not form pseudocystidia but is exceptional in having two types of true cystidia in the hymenium. Macrocystidia and lamprocystidia both occur in Russulaceae but up to now never have been encountered in a single species. Of interest, in *L. ambicystidiatus* the two types of true cystidia are found intermixed in the hymenium, both at the sides and the edge of the lamellae.

*Lactifluus* aff. *leoninus* (Verbeken & E. Horak) Verbeken, Mycotaxon 118:451. 2011. FIGS. 2b, 4.

Basidiocarps middle-sized to large, stout. Pileus up to 9 cm diam, planoconcave with margin slightly incurved, subglabrous, dry, slightly rugose, reddish yellow (4A6) to golden yellow (5B7). Context 3 mm thick in the pileus, cream yellow. Lamellae 8 mm broad, distant, thick, subdecurrent, light yellow (4A4, 4A5) when mature, pale cream colored when young, unchanging. Stipe  $3 \times 1.5$  cm, equal or tapering downward, stout, solid, firm, subpruinose, light yellow (4A4) to light orange (5A4) when mature, paler when young. Latex white, moderately copious, unchanging.

Spores (7.5–)8.0–9.0(–9.5)  $\times$  (6.0–)6.5–7.5(–8.0)  $\mu\text{m}$  ( $Q = 1.14\text{--}1.23[1.29]$ ,  $Q = 1.19 \pm 0.04$ ) (40/1/1), broadly ellipsoid to ellipsoid; ornamentation up to 0.8  $\mu\text{m}$  high, composed of irregular short ridges and warts connected by fine lines, close meshes present but uncommon; plage not amyloid. Basidia 55–65  $\times$  10–12  $\mu\text{m}$ , clavate, four-spored. Macrocystidia absent. Pseudocystidia rare, stout, 7–9  $\mu\text{m}$  diam, cylindrical. Hymenophoral trama with sphaerocytes, lacking typical rosettes. Pileipellis an ixotrichopalisade when mature, more as an ixotrichoderm to ixocutis when young, up to 100  $\mu\text{m}$  thick, composed of cylindrical hyphae 5–10  $\mu\text{m}$  thick and irregularly swollen hyphae up to 17  $\mu\text{m}$  broad; suprapellis with terminal cells 3–5  $\mu\text{m}$  thick with wall 0.5  $\mu\text{m}$  thick. Stipitipellis a cutis with projecting hyphal ends, hyphae 3–5  $\mu\text{m}$  thick, some hyphal ends slightly thick-walled. Lactifers common in trama, stout. Trama of stipe and pileus with abundant rosettes.

*Specimens examined:* CHINA. YUNNAN PROVINCE: Jingdong County, Dachaoshan, 1000 m, on ground in mixed forest with *Pinus kesiya* var. *langbianensis* and fagaceous trees, 3-VII-2012, X.H. Wang 3405 (KUN F75811).

*Notes:* *Lactifluus leoninus* originally was described from Papua New Guinea (Verbeken and Horak 1999). The species was characterized by furcate-sulcate pileus, latex changing to ochraceous, wart-like spore ornamentation, and absence of thick-walled hyphae in the

pileipellis. Although the Chinese sample studied here shares the general characters of *L. leoninus*, the slightly thick-walled hyphae in the uppermost layer of the pileipellis, the unchanging latex and the non-sulcate pileus suggest it might be a different species.

The 28S and *rpb2* sequences of the Chinese sample are identical with those of a Thai sample labeled *L. leoninus* (28S: JN388989, *rpb2*: JN375592; van de Putte et al. 2012). Two Thai environmental samples (Roy et al. 2009) have highly similar ITS sequences (with 99% similarity). These four samples appear to be conspecific. No sequence from the holotype is available. A ITS+28S sequence of an environmental sample from the type locality of *L. leoninus* (Oomsis, Papua New Guinea; Tedersoo and Pölme 2012) suggests a close relationship to the Chinese and Thai samples (with 97% similarity). Multigene sequences from additional collections are needed to determine whether the observed morphological differences between the Chinese sample and the type collection are interspecific.

***Lactifluus subpruinus*** X.H. Wang, sp. nov.  
FIGS. 2c, d, 5a–d

MycoBank MB801947

*Typification:* CHINA. GUANGDONG PROVINCE: Shixing County, Longdouxie, 400 m, under forest of *Castanopsis* spp., 14-IX-2011, X.H. Wang 3131 (KUN F73639, HOLOTYPE).

*Etymology:* Named after the subpruinose pileus.

*Diagnosis:* *L. subpruinus* is recognized by the reddish brown subpruinose pileus, crowded lamellae, latex staining lamellae brownish, and slender thin-walled macrocystidia.

Basidiocarps medium-sized to large, compact. Pileus 5–8 cm in diam, applanate with center depressed; surface subpruinose to pruinose, subvelvety, dry, cracked or not, orange brown to reddish brownish (6D6–7D6); margin radially rugose. Context 3–5 mm thick in the pileus, whitish to pale yellow, stained brownish by latex, mild. Lamellae 3–4 mm broad, cream-colored to yellowish white (2A2), crowded, shortly decurrent, staining brownish by latex. Stipe 2–4  $\times$  1.2–2 cm, equal or tapering downward, stout, solid, subvelvety, paler than the pileus. Latex white, copious, changing to watery, staining lamellae brownish, sticky. Spore print white. With fish-like odor.

Spores 5.5–7.5(–8.0)  $\times$  4.5–6.0(–6.5)  $\mu\text{m}$  ( $Q = [1.13\text{--}]1.15\text{--}1.30[1.35]$ ,  $Q = 1.21 \pm 0.050$  (100/5/5), ellipsoid; ornamentation up to 0.5  $\mu\text{m}$  high, mostly 0.1–0.3  $\mu\text{m}$  high, composed of irregular ridges forming incomplete reticulum; plage not amyloid. Basidia 45–65  $\times$  7–9  $\mu\text{m}$ , clavate, four-spored. Pseudocystidia rare, 3–4  $\mu\text{m}$  diam. Pleuromacrocyti-



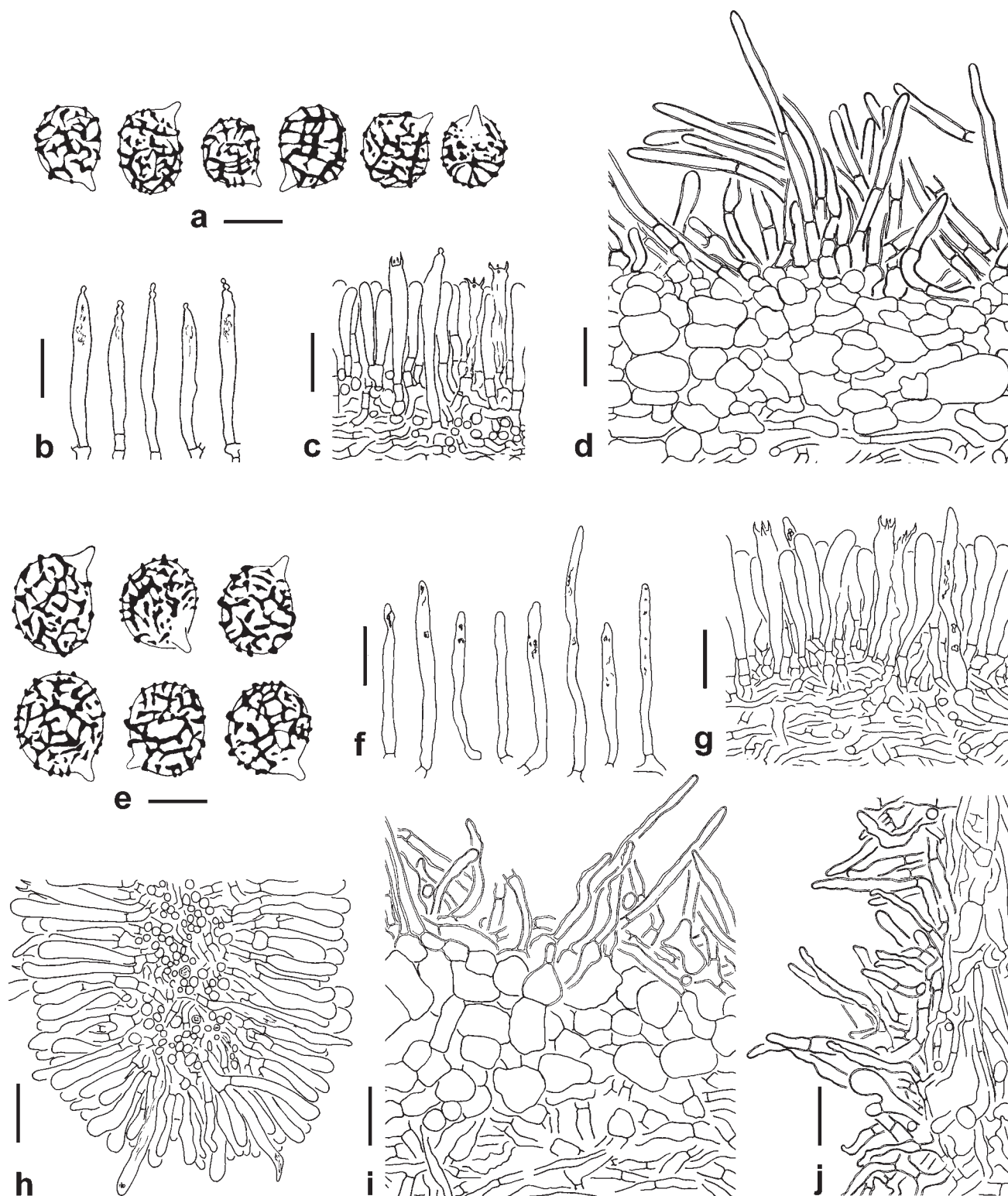


FIG. 5. *Lactifluus subpruinus* (HOLOTYPE). a. Spores. b. Hymenium. c. Pleuromacrocytidia. d. Pileipellis *Lactifluus tropicosinicus* (HOLOTYPE). e. Spores. f. Pleuromacrocytidia. g. Hymenium. h. Lamellar edge. i. Pileipellis. j. Stipitipellis. Bars: a, e = 5  $\mu$ m; b-d, f-j = 25  $\mu$ m.

dia 50–90  $\times$  (5–)6–8(–9)  $\mu\text{m}$ , subcylindrical, apex often moniliform, with or without sparse content, embedded in hymenium, not projecting beyond the layer of basidia or projecting 10–20  $\mu\text{m}$ , arising either from the same level as basal septa of basidia or deeply from the subhymenium or the hymenophoral trama. Lamellar edge sterile; cheilomacrocytidia common in young basidiocarps, rare or absent with age. Hymenophoral trama with sphaerocytes, lacking typical rosettes. Pileipellis a lampropalisade; hyphae in suprapellis slightly thick-walled (mostly 0.5  $\mu\text{m}$  thick, rarely 1.0  $\mu\text{m}$  thick), septate, mostly 50–100  $\mu\text{m}$  long and 5–7  $\mu\text{m}$  thick; subpellis 60–100(–130)  $\mu\text{m}$  thick, composed of isodiametric cells 15–30  $\mu\text{m}$  diam. Stipitipellis a trichoderm to slightly lamprotrichoderm; sometimes an oedotrichoderm; hyphae in suprapellis thin-walled to slightly thick-walled, 20–40  $\times$  5–6  $\mu\text{m}$ , some inflated to 8  $\mu\text{m}$  wide, cylindrical, locally ventricose, often branching; cells in subpellis cylindrical or inflated to 15  $\mu\text{m}$  diam. Lactifers common, robust. Trama of pileus and stipe with abundant rosettes.

*Specimens examined:* CHINA. ANHUI PROVINCE: Qian-shan County, Mt. Tianzhu, 680 m, under mixed forest of *P. massoniana* and fagaceous trees, 29-VIII-2011, X.H. Wang 3036 (KUN F73579); FUJIAN PROVINCE: Sanming, Sanyuan National Forest Park of *Castanopsis kamakamii*, 200 m, 25-VIII-2007, Y.C. Li 1011 (KUN F53356); GUANGDONG PROVINCE: Fengkai, Heishiding Nature Reserve, 22-V-2012, F. Li 330 (KUN F75812); SICHUAN PROVINCE: Pujiang County, Daxing, 30°14'N, 103°25'E, 600 m, on ground, in forest of *Pinus massoniana* and *Camellia* sp., 6-VII-1997, P.Q. Sun 2797 (KUN F31210); YUNNAN PROVINCE: Puer Prefecture, Simao District, Caiyanghe Forest Park, 1400 m, 6-VII-2012, X.H. Wang 3489 (KUN F76034); Puer prefecture, Simao District, near Xinfang Reservoir, 1400 m, 6-VII-2012, X.H. Wang 3514 (KUN F76053); Yulong County, Shitou, Liju village, 2600 m, 21-VIII-2008, Q. Zhao 282 (KUN F55259).

*Notes:* This new species is morphotype 3 of *L.* sect. *Tenuicytidiati* (FIGS. 1, 5). It is well separated from the other two species (i.e. *L. tropicosinicus* and *L. tenuicytidiatus*) by the orange-brown to reddish brown pileus and crowded lamellae. In the field the species could easily pass as *L. volemus* sensu lato. Nevertheless, the ellipsoid spores with an incomplete reticulum and the thin-walled macrocytidia, sometimes together with a subpruinose pileus, easily distinguish *L. subpruinus* from all other members of the *L. volemus* complex. One sample (GenBank accession number AF354455) listed as *L. volemus* by Manassila et al. (2005) from northern Thailand seems close to *L. subpruinus* based on analysis of ITS sequences (result not shown). With low similarity to the ITS sequence of *L. subpruinus* (95%), the Thai sample might represent a distinct species.

***Lactifluus tropicosinicus*** X.H. Wang, sp. nov.  
FIGS. 2f, 5e–j

MycoBank MB811230

*Etymology:* Referring to the geographical origin, from tropical China.

*Typification:* CHINA. YUNNAN PROVINCE: Baoshan, forest along state road 322 from Baoshan to Yongping, 2030 m, 25°29.713'N, 99°39.02'E, in mixed forest of *Castanopsis* sp., *Pinus yunnanensis*, and *Quercus* sp., 30-VII-2009, Y.C. Li 1879 (KUN F59627, HOLOTYPE).

*Diagnosis:* *L. tropicosinicus* is highly similar to *L. tenuicytidiatus* but differs in the less distant lamellae.

Basidiocarps medium-sized to big, compact, fragile, thick-fleshed. Pileus 6–11 cm diam, concave to shallowly infundibuliform, center papillate or not, subvelvety to velvety, dry, slightly to strongly rugose, yellowish white to range yellow. Context 4–6 mm thick in the pileus, pale yellow. Lamellae 4–8 mm broad, sub-crowded to sub-distant, straight to shortly decurrent, yellowish white (paler than 3A3–4A3), pale cream-colored when young, stained brownish. Stipe 4–7  $\times$  1–2 cm, equal or slightly tapering downward, solid, firm, subglabrous, yellowish white to nearly whitish. Latex white, copious, mild, staining lamellae brownish, sticky. Spore print white. With fish-like odor.

Spores (6.0–)6.5–8.0(–9.0)  $\times$  (5.0–)5.5–6.5(–7.5)  $\mu\text{m}$  ( $Q = [1.08–]1.15–1.27[–1.40]$ ,  $Q = 1.20 \pm 0.06$ ) (130/5/5), broadly ellipsoid to ellipsoid; ornamentation up to 0.5  $\mu\text{m}$  high, mostly 0.1–0.3  $\mu\text{m}$  high, composed of irregular short ridges and warts connected by fine lines, not forming an reticulum, but closed meshes present; plage not amyloid. Basidia 55–73  $\times$  7–10  $\mu\text{m}$ , clavate, four-spored. Pseudocyttidia scarce, more common close to lamellar edge, 3–5  $\mu\text{m}$  diam, slender, cylindrical. Pleuromacrocyttidia scarce, rarely common, (55–)70–90  $\times$  (5–)6–9  $\mu\text{m}$ , subcylindrical, rarely sublanceolate, apex obtuse, rarely almost moniliform, with sparse needle-shaped content, embedded in hymenium, projecting beyond the layer of basidia or not, arising either from the same level as basal septa of basidia or deeply from the subhymenium. Lamellar edge sterile; cheilomacrocyttidia nearly common, similar to pleuromacrocyttidia in shape but smaller. Hymenophoral trama with sphaerocytes, lacking typical rosettes. Pileipellis a lampropalisade; hyphae in suprapellis 50–70(–120)  $\times$  4–5  $\mu\text{m}$ , with wall 0.5–1.0  $\mu\text{m}$  thick, unevenly thickened, locally with wall to 1.5  $\mu\text{m}$  thick, often less thick-walled at apex, septate, obtuse at apex; subpellis 100–150  $\mu\text{m}$  thick, composed of isodiametric cells 15–35 (–45)  $\mu\text{m}$  diam. Stipitipellis a lamprotrichoderm; terminal cells 30–70  $\mu\text{m} \times$  4–6(–7)  $\mu\text{m}$ , slightly thick-walled.

Lactifers common, robust. Trama of pileus and stipe with abundant rosettes.

*Specimens examined:* CHINA. YUNNAN PROVINCE: Baoshan, forest along state road 322 from Baoshan to Yongping, 2030 m, 25°29.713'N, 99°394.02'E, in mixed forest of *Castanopsis* sp., *Pinus yunnanensis* and *Quercus* sp., 30-VII-2009, Y.C. Li 1878 (KUN F59626); Y.C. Li 1879 (KUN F59627, HOLOTYPE), L.P. Tang 1011 (KUN F56968), Q. Cai 52 (KUN F58719); Kunming, Mushuihua Wild Mushroom Market, 30-VII-2013, X.H. Chen KM1 (KUN F83764); Tengchong, Houqiao, 10-VIII-2011, X.T. Zhu 477 (KUN F75765).

*Notes:* This is one of the two phylogenetic species under morphotype 1 in *L. sect. Tenuicystidiati* (FIG. 1). The other phylogenetic species under morphotype 1 (as "*Lactifluus* sp.") cannot be separated morphologically from this new species in the field, but microscopically the three collections, XHW3449, 3450 and 3451, lack macrocystidia. It is interesting to note that the singleton of XHW3512, which shows clear genetic diversification from the three collections above, has macrocystidia. However, if we follow the exhaustive classification in GCPSR (Dettman et al. 2003), the singleton of XHW3512 cannot be recognized as a separate species. Lacking sufficient samples, we tentatively leave the other phylogenetic species unnamed.

Compared with *L. tenuicystidiatus* (or the collections of morphotype 2), this new species has more crowded lamellae, less stout basidiocarps and less common macrocystidia. The morphological differences between the two species, however, are not always clear. The spacing of the lamellae and the general appearance of the holotype of *L. tenuicystidiatus* are intermediate between most typical *L. tenuicystidiatus* and *L. tropicosinicus*. Re-examination of the collections cited under *L. tenuicystidiatus* when the species was published showed that they actually included at least collections of *L. tenuicystidiatus*, *L. tropicosinicus*, "*Lactifluus* sp." and *L. aff. tenuicystidiatus* (in FIG. 1). In the local markets in Yunnan and subtropical-tropical China, collections with a general morphology of *L. tropicosinicus* and *Lactifluus* sp. (morphotype 1) are more commonly encountered than morphotype 2. All these species are popular, wild, edible mushrooms in southern China.

**Lactifluus sect. Ambicystidiati** X.H. Wang, sect. nov.  
MycoBank MB801948

Basidiocarps big, stout, pale. Pileus and stipe velvety. Hymenophoral cystidia present as two kinds: thin-walled macrocystidia and thick-walled lamprocystidia. Lactiferous system poorly developed, only as remnants of lactifers. Pseudocystidia absent. Spores ellipsoid, with warts and short ridges rarely connected. Pileipellis a lamprooedotrichoderm.

*Type:* *Lactifluus ambicystidiatus* X.H. Wang

*Notes:* The placement of *L. ambicystidiatus* in Clade A by molecular data is unexpected. The general morphology of this species is strongly reminiscent of species in *L. sect. Albatii*, placed in clade B in this study. Within Clade A, five groups are recognized based on topology (FIG. 1) and morphological features. The morphological delimitations among these five groups are clear. The whitish basidiocarps of *L. ambicystidiatus* are similar to those of *L. subg. Piperati* Verbeke, but the thick-walled hairs in the pileipellis and stipitipellis and the lamprocystidia clearly distinguish it. Lamprocystidia also are present in *L. sect. Lactifluus*, but *L. ambicystidiatus* differs in the whitish basidiocarps, pileipellis without a layer of isodiametric cells, ellipsoid spores with low ornamentation, and presence of macrocystidia. The long branch leading to the *Ambicystidiatus* lineage and the strong support excluding it from *L. sect. Lactifluus*, *L. sect. Tenuicystidiati* and *L. subg. Gerardii* suggest it should be recognized as an independent infrageneric taxon. Although this new section is in the major clade that includes the type of the genus, for the time being, we do not assign it into *L. subg. Lactifluus* because the subgenus is in high need of revision and *L. ambicystidiatus* is excluded from the monophyly formed by *L. sect. Lactifluus* and *L. sect. Tenuicystidiati* with significant support. It is left "incertae sedis" within *Lactifluus*.

**Lactifluus sect. Tenuicystidiati** X.H. Wang & Verbeke, sect. nov.

MycoBank MB801949

Basidiocarps medium-sized to large, stout. Pileus and stipe subvelvety, often with orange tinge. Pileipellis a lampropalisade. Hymenophoral macrocystidia thin-walled, slender. Spores ellipsoid, with low ornamentation more or less connected. Latex staining lamellae brownish. With fish-like odor.

*Type:* *Lactarius tenuicystidiatus* X.H. Wang & Verbeke, Nova Hedwigia 83:173. 2006.

*Notes:* The type species of this section was tentatively put in *L. sect. Pseudogymnocarpi* by Wang and Verbeke (2006), based on the morphological similarity to some species of that section. It was left as an open question whether the species with thin-walled macrocystidia would form a separate group. The molecular data presented here does not support the taxonomic assignment above (*L. sect. Pseudogymnocarpi* is nested within clade B) but suggests that the *Tenuicystidiatus* lineage is a sister group to *L. sect. Lactifluus* s. str. (*L. volemus* complex) in clade A. The subclade formed by the *Tenuicystidiatus* lineage and *L. sect. Lactifluus* s. str. is one of the four major molecular subclades. Although the two groups share



a few morphological features, such as orange tinge of the pileus, brownish staining latex, fish-like odor and lamprotrichopalisade as the pileipellis, the morphological differences between them are clear: the smaller ellipsoid spores with a fine and incomplete reticulum and the slender thin-walled macrocystidia in the *Tenuicystidiatus* lineage are in contrast with the bigger globose spores with complete reticulum and lamprocystidia in *L. volemus* complex. By recognizing the *Tenuicystidiatus* lineage as a new section these differences will be clearly displayed. Although *L.* subg. *Lactifluus* is in high need of revision, we assign this section to *L.* subg. *Lactifluus* because it is supported to be the closest relative of *L.* sect. *Lactifluus* and the two sections do share some characters. Up to now four collections were found to lack macrocystidia in this section. This may merely represent an occasional variation in some individuals.

#### DISCUSSION

*Delimitation of Lactifluus and diversity of morphological characters in the genus.*—The morphological diversity within *Lactifluus* is mainly seen in the different types of pileipellis and stipitipellis, presence/absence of hymenophoral macrocystidia and lamprocystidia, and shape and ornamentation of spores and general appearance of basidiocarps (Verbeken et al. 2001). New representatives discovered from the tropics in recent years present new combinations of these individual characters (van de Putte et al. 2009; Miller et al. 2012; Wang et al. 2006, 2012; Morozova et al. 2013). In this particular study species in *L.* sect. *Tenuicystidiati* share phenotypic features with species of *L.* sect. *Lactifluus*, such as the browning latex, fish-like odor and lamprotrichopalisade as the pileipellis structure. With species of *L.* sect. *Rugati* they share the smaller lowly ornamented ellipsoid spores and pileipellis structure; and with *L. ochrogalactus* they share the slender macrocystidia and pileipellis structure. More intriguing, *L. ambicystidiatus*, one of the new species described here, possesses basidiocarps with two types of true cystidia, macrocystidia and lamprocystidia. This is exceptional in *Lactifluus* and even in the family Russulaceae. It appears that at least in tropical Asia our present knowledge of the morphological diversity of *Lactifluus* is still poor.

Although some *Lactifluus* species are highly similar to *Russula*, the presence of pseudocystidia (i.e. the terminal parts of lactiferous hyphae that are ascending in the hymenium) combined with a well-developed lactiferous network, have been thought to distinguish *Lactarius* and *Lactifluus* from *Russula* (Buyck 1999). Buyck et al. (2007) and Verbeken

(2001) found that some species of *Lactifluus* lack latex but a lactiferous system was still well developed and pseudocystidia present. Wang et al. (2012) failed to find pseudocystidia in *L. parvigerardii*, but the lactiferous system was still well developed. *Lactifluus ambicystidiatus*, however, entirely lacks both a ramified lactiferous system in every tissue of the basidiocarps and pseudocystidia in the hymenium. The lactiferous system in *Lactifluus* thus ranges from a very well developed system in most species to very few or no lactiferous hyphae (only remnants) in others, in combination with common pseudocystidia, rare or absent with age. *Lactifluus* thereby becomes the second genus in Russulaceae, after *Multifurca*, where such wide variation is seen in the lactiferous system. This blurs the morphological delimitation between *Lactifluus* and *Russula*.

*Phylogenetic and taxonomic significance of the three new lineages.*—This study is the first to revisit the phylogeny of *Lactifluus* since Buyck et al. (2008), with multiple genes and reference to traditional infrageneric classification. Using our new molecular data and those by Stubbe et al. (2010), van de Putte et al. (2010) and Morozova et al. (2013), this study is able to make the *Lactifluus* phylogeny more complete. In the phylogeny presented by Buyck et al. (2008), three well-supported major clades were obtained, represented by samples of north temperate *L. volemus*-*L. piperatus* (mostly equivalent to Clade A in FIG. 1), tropical African *L. rubroviolascens*-*L. longisporus* and temperate and African *L.* subg. *Lactariopsis*-*L.* sect. *Edules* (two of the three major subclades of Clade B in FIG. 1). Stubbe et al. (2010) added North American-Australasian *L.* subg. *Gerardii* to the *L. volemus*-*L. piperatus* clade and presented a new major clade formed by *L. clarkeae*, *L. panuoides* and *L. chiapanensis*. The same four major clades are retrieved in this study. With our new data added, the major clade that includes the type of *Lactifluus* (equivalent to clade A in FIG. 1) now comprises two additional subclades here adopted as sections. One of these, *L.* sect. *Tenuicystidiati*, forms a sister group to *L.* sect. *Lactifluus*, which also makes sense morphologically. This questions the closest relationship between *L.* sect. *Lactifluus* and *L.* subg. *Gerardii* as suggested by Stubbe et al. (2010). The present molecular data did not significantly resolve the affinity of *L.* sect. *Ambicystidiati* to the other sections of Clade A. Nevertheless *L. ambicystidiatus* is unique within Clade A in lacking a distinct sublayer of isodiametric cells in the pileipellis, which is shared by all of the other members (Verbeken 2001, Stubbe et al. 2010, van de Putte et al. 2012, this study). Within clade A *L.* sect. *Lactifluus* and *L. ambicystidiatus* share lamprocystidia. The presence of both lamprocystidia and macro-

cystidia in *L. ambicystidiatus* hopefully will provide useful data to reconstruct the character evolution in *Lactifluus*. The absence of a lactiferous network in this species might be a loss in character evolution.

This study adds further evidence to studies that have shown that the two biggest subgenera of *Lactifluus*, *L.* subg. *Lactifluus* and *L.* subg. *Lactariopsis*, are paraphyletic (Buyck et al. 2007, 2008; Stubbe et al. 2010; van de Putte et al. 2012). *Lactifluus* sect. *Phlebonemi*, which was placed into *L.* subg. *Lactifluus* (Verbeken 2001, Verbeken et al. 2012), is supported to be distant to *L.* sect. *Lactifluus* in this study and forms an independent major clade with American *L. chiapanensis* and *L. clarkeae* from Oceania (part of *L.* sect. *Tomentosi*). *Lactifluus leoninus*, which represents the *Leoninus* lineage, was thought to be a member of *L.* sect. *Chamaeleontini* due to the unveiled basidiocarps and pileipellis composed of thin-walled extremities (Verbeken and Horak 1999). However, our analyses show that the *Leoninus* lineage is sister to *L.* subg. *Lactariopsis* s.str., which taxonomically covered *L.* sect. *Lactariopsis* and *L.* sect. *Chamaeleontini* (Verbeken 1998b, Verbeken et al. 2012). Using multilocus data, this study confirmed the conclusion of Buyck et al. (2007) that *L.* sect. *Chamaeleontini* is not monophyletic. Homoplasy within *L.* subg. *Lactariopsis* seems to be prevalent.

Overall it appears that with more new members included in the phylogeny of *Lactifluus* it becomes harder to define many of the major evolutionary lineages morphologically. For instance, *L. ambicystidiatus* is phenotypically much more similar to *L.* sect. *Albati* (in Clade B) than to the other members of Clade A. Also the major clade formed by *L.* aff. *luteolus*, *L. chiapanensis*, and *L. clarkeae* does not seem to show clear morphological convergence. Although the *Lactifluus* phylogeny is still premature, it can be seen that only shallow clades (i.e. species, species complexes or sections) are well supported by morphological features. The relatively long branches leading to these clades suggest they have been separated for a long time or that the taxon sampling is still incomplete.

Recent studies on some species complexes of *Lactifluus* in subtropical-tropical Asia have revealed high species diversity (Stubbe et al. 2010; van de Putte et al. 2010, 2012; de Crop et al. 2014). After the *L. volemus*, *L. gerardii* and *L. piperatus* species complexes, the *L. tenuicystidiatus* complex reported in this study represents another group rich in phylogenetic species in subtropical-tropical Asia. Moreover, the clear genetic diversification within *L. luteolus* suggests it might comprise different phylogenetic species in Asia. The two *Lactifluus* species with pleurotoid habit reported by Morozova et al.

(2013) are confirmed in this study to have important taxonomic and phylogenetic implications. *Lactifluus ambicystidiatus* shows unexpected morphological diversity within the genus. The *Leoninus* lineage presents a new link between species from tropical Asia and tropical Africa. The diversity of *Lactifluus* in Asia, comprising both morphologically distinct species and cryptic species, appears to be much higher than was thought. The above studies and some other studies on different groups of fungi in the same region (Feng et al. 2012, Li et al. 2010, Li et al. 2011, Halling et al. 2012, de Crop et al. 2014a) suggest that subtropical-tropical Asia will be a key region assessing the actual species diversity in *Lactifluus* and therefore will contribute greatly to a better understanding of the evolution and distribution of this genus.

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